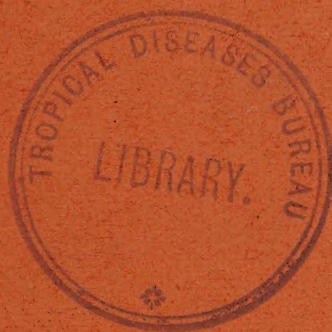
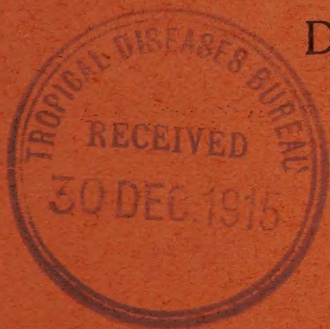


The Culture of Leishmania from the Finger-
Blood of a Case of Indian Kala-Azar, with
some Remarks on the Nature of certain
Granular Bodies recently described from this
Disease.



BY

C. M. WENYON, M.B., B.S., B.Sc.

Protozoologist to the London School of Tropical Medicine.

[Reprinted from "The Journal of Tropical Medicine and Hygiene,"
February 16, 1914.]

Vol. 17

LONDON
JOHN BALE, SONS & DANIELSSON, LIMITED
OXFORD HOUSE
83-91, Great Titchfield Street, Oxford Street, W
1914



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Repeated examinations of the peripheral blood had failed to reveal any parasites, though these had previously been demonstrated by liver puncture. On January 20 six tubes of N.N.N. medium were inoculated, each with two to three drops of blood, obtained by pricking the sterilized finger of the patient. The tubes were then incubated at a temperature of 23° - 25° C. Examined on the sixth and eleventh days no growth of flagellates was noted in the tubes, though one was contaminated with bacteria. The tubes were again examined on the eighteenth day, and in each of the five tubes free from bacteria flagellates were present in such numbers as to be easily seen with the low power objective. It is unfortunate that the peripheral blood of the patient was not examined for leishmania on the day the tubes were inoculated, but the long time intervening between the inoculation of the tubes and the appearance of flagellates in these in sufficient numbers to be detected proves that the leishmania must have been present in the blood in very small numbers. This development and multiplication of the leishmania in the test-tube is a practical demonstration of the possibility of the true invertebrate host becoming infected from the peripheral blood of cases of kala-azar.

Some time ago I was able to obtain a culture of flagellates from a case of aural ulceration, which was under the care of Professor W. J. Simpson. The patient, an Englishman, had an ulcer on the margin of the ear, which had appeared after a journey in South America. This had persisted for about two years, and had resisted the various treatments employed. I made smears from scrapings from the base of the ulcer, as well as from material obtained by puncture of the red margin. At the same time the red raised skin forming the edge of the ulcer was

sterilized with alcoholic iodine solution. This was punctured with a needle, and material was obtained for inoculating three tubes of N.N.N. medium by inserting a fine glass pipette through the puncture wound. Prolonged examination of the various smears failed to reveal any leishmania, but after an interval of three weeks flagellates were present in one of the tubes inoculated, thus proving that leishmania had been present in the ulcer, and confirming the suspicion that the case was one of dermal leishmaniasis.

The culture method on N.N.N. medium can thus be used as a means of diagnosis in leishmania diseases, especially in cases of kala-azar, where spleen or liver puncture cannot be undertaken, and where one has failed to find leishmania in the peripheral blood. In some cases, as in the case of Oriental sore just mentioned, a diagnosis was made in this way alone. In animal experiments it is now a fairly common experience to obtain a culture from organs which have failed to reveal leishmania in stained smears. In many of these cases a more prolonged and careful examination of stained films might reveal the organism, but one rarely has time to devote many hours to such a search. In N.N.N. medium a single parasite may become a flagellate, and multiply in the course of a week or two till many thousands are present. In such cases the actual time spent on making the diagnosis is comparatively small. The only difficulty is with the N.N.N. medium, which may be a hindrance to anyone not accustomed to work with it. The medium itself is easy enough to prepare, and can be made by any laboratory assistant versed in bacteriological technique; 14 grammes of agar, 6 grammes of salt, and 900 c.c. of distilled water are dissolved in the usual manner, and distributed without filtering in test-tubes (1 inch in each tube). The tubes are plugged

and sterilized in the autoclave. A rabbit is killed with chloroform, and tied out on its back. The thorax is painted with solution of iodine, and the heart exposed with sterile instruments. A sterile 20 c.c. syringe, with large needle, is used for drawing the blood directly from the heart. The agar tubes, which have been cooled to below 50° C., and in which the agar is still liquid, are held and opened by an assistant, and into each is introduced about 1 c.c. of blood, which has just been drawn from the rabbit's heart. The tubes are rolled in the hand to mix the still liquid agar and blood and sloped. When solid they are incubated at 37° C for twenty-four hours, to determine their sterility, when they are ready for use. Inoculation is made into the water of condensation which has collected in the tubes. They are then incubated at a temperature of 22° - 25° C., after being covered with a rubber cap to prevent evaporation. By drawing the blood from the heart of a killed animal it is possible to obtain sufficient to make a dozen to twenty tubes of medium. If, however, the operation is performed while the animal is still alive under anæsthesia, much more blood can be obtained, as the heart is still pulsating, and fills with blood after each quantity drawn off. In this case it is better to transfer the blood to small flasks containing beads for defibrination before distribution in the agar tubes. In order to watch the progress of the growth in the tubes, all that is necessary is to remove a small quantity of the liquid on a platinum loop, place it on a slide, and examine it with the $\frac{2}{3}$ or $\frac{1}{8}$ in. objective, with the condenser down. There is no need to cover with a cover-glass, as with a little experience the flagellates can easily be seen swimming about with the $\frac{2}{3}$ in. objective. Care must be taken to prevent bacteria gaining access to the tubes.

In cases of kala-azar and Oriental sore, in which prolonged search for leishmania in stained films has been negative, and in which the culture method has given a positive result, are we to suppose that the culture has resulted from some stage of the parasite not hitherto recognized, or from leishmania themselves, which have been present in numbers too small to be detected? In the Sudan, Archibald (*Journal of the Royal Army Medical Corps*, May, 1913) discovered in the spleen and liver of a case of kala-azar blue staining protoplasmic masses containing purple staining granules in varying number, but no leishmania. A monkey inoculated from the organs developed kala-azar with definite leishmania in its organs. It was suggested that the granular masses seen in the human subject might represent some hitherto unrecognized stage of the leishmania. More recently Statham and Butler (*Journal of the Royal Army Medical Corps*, December, 1913) have described similar bodies from the liver of a case of splenic enlargement in West Africa, and they suggest that they possibly represent the schizogonic stage of some protozoon, and in the light of Archibald's observation that kala-azar may exist in West Africa, and that these bodies are developmental stages of leishmania. The typical leishmania, however, could not be found in the smears. In the same journal Smallman describes similar bodies from the liver of a case of Mediterranean kala-azar. At this time no leishmania were found in the eight smears, though previously they had been found, so the author inclines towards the view that some stage in the development of leishmania is represented.

It might, therefore, be urged that it is some such a stage which is responsible for the appearance of flagellates in cultures when examination of smears

has been negative. A very strong argument against this view is the fact that in all these cases it requires a long interval of two to three weeks for the flagellates to appear in the tubes in sufficient numbers to be detected. When the leishmania inoculated into the tubes are numerous, flagellates are present in great numbers in less than forty-eight hours, so that for any individual leishmania to reach the flagellate stage this length of time is necessary. The flagellates then multiply and increase in numbers. When the leishmania introduced are very few it may safely be assumed that they also become flagellates in about forty-eight hours, but that it is only after two or three weeks of multiplication that they are in sufficient numbers to be recognized.

Now, as regards the bodies which have been described by the various observers just quoted, no evidence whatever has been produced to prove their parasitic nature. Because protozoa consist of protoplasm and chromatin, it must not be forgotten that the cells of the higher animals consist of the same two substances, and in smears stained with Romanowsky stains the cytoplasm, whether of a protozoon or the cell of a higher animal, tends to stain blue, while the chromatin (and other granules also) stains varying shades of red and purple. And because certain stages of some protozoa are cytoplasmic bodies, containing many chromatin granules, it must be remembered that any portion of cytoplasm containing granules may give the same appearance if treated and stained in the same manner, for protoplasm is essentially the same wherever it occurs. Such simulation may give rise to error, and one must not be too hasty in concluding that appearances of this kind in smears of organs are produced, or are most likely to be produced, by parasitic protozoa.

For some time I have been working with experimental leishmaniasis in animals, and have been in the habit of performing liver punctures on these. On several occasions, in both dogs and rats, I have encountered in these liver smears the bodies described by the authors mentioned above. The bodies vary considerably. Sometimes they are exact reproductions of Archibald's coloured plate, at others they cannot be distinguished from those figured in the papers of Statham and Butler, and Smallman. I was at first surprised at this, though I did not think it possible that they represented any stage of development of leishmania. Accordingly, I checked the result by examining in the same manner the livers of other uninoculated dogs and rats. I found that exactly the same bodies might occur in these, so that any possibility of their being connected with the leishmania was at once done away with. The uninoculated animals were in perfect health, as, indeed, were most of the others which had been previously inoculated with leishmania, so that I doubt very much if the protoplasmic bodies with staining granules are of any pathological significance whatever. After careful examination of many films, I am quite convinced that the bodies in question are merely detached portions of the cytoplasm of large cells which are themselves charged with granules. The exact staining of the cytoplasm varies very much, and depends largely on the extent of disintegration of the cells and the amount of flattening to which they have been submitted in the process of film making. The detached portions of cytoplasm, being much thinner and more spread out, often stain differently to the cytoplasm of the intact cell. In cases of actual kala-azar spleen smears often show cytoplasmic masses, containing varying numbers of leishmania. These are known to

be detached portions of the cytoplasm of the large macrophages, which are themselves filled with leishmania. In a similar manner the large granular cells of any organ may be broken in film making, giving rise to detached portions of cytoplasm containing granules which stain by Romanowsky stains. This is what has occurred in the case of the animals I have examined, and what I feel sure has happened in the cases described by Archibald, Statham and Butler, and Smallman. It may be that in some cases the granules represent a degeneration of some of the liver cells, but one must never forget that they may represent a purely physiological process, and be connected with digestion, in which the liver plays such an important part. The fact that in perfectly healthy animals such bodies occur is distinctly in favour of this view. Another point which must not be forgotten is that many kinds of material other than chromatin are coloured red with the Romanowsky stain and its modifications, so that one must not be too hasty in jumping to the conclusion that every red staining granule is chromatin. In the case of the bodies now under discussion it seems to me that the red staining granules they contain are probably deposits of some substance quite different from chromatin. But, whatever be the nature of the granules, the bodies themselves certainly represent no stage of schizogony of a protozoal organism, since they are derived from large cells from which they have become detached. Therefore, in Archibald's case mentioned above, in which these bodies occurred, and from which a monkey was inoculated and developed kala-azar, I think the only possible conclusion is that the monkey became infected from actual leishmania, which were present in the human being in numbers too small to be detected microscopically. This supposition is sup-

ported by the fact that the patient recovered without any treatment, so that the infection was, at any rate, a mild one. If the granules had represented leishmania, the liver must have been heavily infected. Similarly in the case of dermal leishmaniasis from which I obtained a culture, the flagellates resulted from leishmania actually present rather than from any granular stage undetected by me. Of three tubes of medium inoculated from the sore, it was only in one that flagellates appeared, and this after an incubation of three weeks.

It might have been advisable to illustrate in a coloured plate the bodies found by me in the livers of the animals, but this would have meant nothing more than a reproduction of the admirable plates accompanying the papers of the authors, who have described the bodies from human cases. A reference to these plates will show the bodies which I have found in the liver smears of both the healthy and experimental animals. They are certainly not parasites, and are derived from large cells, as already explained; but on the exact nature of the red staining granules in these cells I am not in a position to pronounce a definite opinion, though I do not think anyone could urge that they themselves are parasitic.



